

# Knockout of SIRT4 decreases chemosensitivity to 5-FU in colorectal cancer cells

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**Abstract.** Previous studies demonstrated that sirtuin (SIRT) 4 is aberrantly expressed in human malignant tumors and is associated with poor prognosis in patients with colorectal cancer. However, the role of SIRT4 in the progression of human colorectal cancer (CRC) and in chemotherapy remains unclear. In the present study, the expression of SIRT4 in CRC tissues and the effect of SIRT4 on colorectal cancer proliferation, migration and invasion was investigated. Additionally, the effects of SIRT4 on the chemosensitivity in colorectal cancer cells and the underlying molecular mechanisms were also explored. The results demonstrated that SIRT4 expression is significantly downregulated in CRC tissues and cell lines. Downregulation of SIRT4 significantly increased tumor proliferation, migration and invasion. Additionally, downregulation of SIRT4 decreased the chemosensitivity of CRC cells by inhibiting cell apoptosis. Thus, these results suggest that SIRT4 may be a promising therapeutic target in CRC.

## Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-associated mortality (1). Despite advances in the diagnosis and therapy, CRC remains a major global health problem (2). Thus, it is necessary to explore the molecular mechanisms underlying the progression of CRC.

Sirtuins (SIRT) 1-7 are a class of nicotinamide adenine dinucleotide (NAD)<sup>+</sup>-dependent deacetylases (3), involved in cellular processes (4), including metabolism (5), stress response (6,7), genomic stability (8,9) and longevity (10). Among SIRTs, SIRT1, SIRT6 and SIRT7 are localized in the nucleus (11-13), SIRT2 resides within the cytoplasm (11), whereas SIRT3, SIRT4 and SIRT5 are mitochondrial (14,15).

Aberrant expression of SIRTs is associated with the pathogenesis of several human diseases, including cancer (16). SIRT4 does not have a NAD<sup>+</sup>-dependent deacetylase activity but downregulates glutamate dehydrogenase activity (17), an enzyme that converts glutamate to  $\alpha$ -ketoglutarate and regulates the metabolism of glutamine and glutamate to ultimately produce ATP (18). SIRT4 regulates the balance between fatty acid oxidation and lipid synthesis in skeletal muscle cells and adipocytes (19,20), and regulates insulin secretion in human pancreatic  $\beta$  cells (17,21). Additionally, SIRT4 serves a role in the metabolism of amino acids (22). In tumor cells, SIRT4 may negatively regulate cell proliferation by inhibiting the uptake of glutamine (23). Jeong *et al* (24) reported that SIRT4 regulates the cellular metabolism in response to DNA damage by inhibiting mitochondrial glutamine metabolism. Additionally, decreased protein levels of SIRT4 have been detected in endometrial adenocarcinoma tissues and are associated with advanced American Joint Committee on Cancer (AJCC) stages (25). Downregulation of SIRT4 is associated with poor prognosis in esophageal squamous cell carcinoma (26). Numerous studies demonstrated that SIRT4 is downregulated in several types of human cancer, including breast (27), gastric (28), liver (29), colon (30) and bladder cancer (31) and leukemia (32), however, primarily in lung cancer (33,34). Although previous studies suggested that SIRT4 may act as a tumor suppressor (35), the function of SIRT4 in human cancer remains unclear.

In the present study, the expression of SIRT4 was detected in CRC tissues. Additionally, the effect of SIRT4 downregulation on colon cancer proliferation, migration and invasion was investigated. The effects of SIRT4 on the chemotherapeutic sensitivity of CRC cells and the underlying molecular mechanisms were also explored. The present study provides insights on the role of SIRT4 in CRC progression.

## Materials and methods

**Cell lines and cell culture.** All cell lines were provided by the Shanghai Institute of Cell Biology (Shanghai, China). The human CRC cell lines HCT116, SW1116, SW620 and DLD1 were maintained in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel) and the normal colorectal cell line FHC were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences,

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Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Tissue samples.** A total of 30 tissues (15 CRC and 15 matched adjacent normal tissues of male patients) were obtained from the Department of Gastrointestinal Surgical Oncology at Harbin Medical University Cancer Hospital (Heilongjiang, China) between 2011 and 2012. The range years was 38-76 years and the mean age of patients was 52 years. None of the patients received chemotherapy before surgery. All patients signed written informed consent, and the study was approved by the Ethics Committee of Harbin Medical University (Heilongjiang, China).

**The Cancer Genome Atlas (TCGA) database.** The SIRT4 expression data of 174 samples were downloaded from TCGA data portal on August 2016 (<https://cancergenome.nih.gov/>). According to the TCGA barcode, the expression data was divided into CRC data and normal data, including 155 CRC tissues and 19 normal colon tissues. The statistical analyses and figures were performed by GraphPad Prism software 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) based on two sets of expression data.

**Immunohistochemistry and evaluation of staining.** Tissue sections (3.5  $\mu$ m thick) were prepared from paraffin-embedded tissues. Briefly, sections were deparaffinized in xylene for 5 min (four times) and rehydrated in a descending ethanol series at room temperature. Antigen retrieval was performed using a pressure cooker for 3 min at 121°C in Tris-EDTA buffer. Endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide for 10 min at room temperature. The sections were incubated with primary antibody against SIRT4 (ab10140; 1:100; Abcam, Cambridge, UK) overnight at 4°C. Following the primary incubation, sections were incubated with secondary antibody solution (ZB-2306; 1:5,000; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. The sections were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB; ZSGB-BIO; OriGene Technologies, Inc.) and counterstained with hematoxylin. Tissues stained with PBS instead of primary antibody, served as negative controls.

The histological evaluation was performed by two pathologists from Harbin Medical University Cancer Hospital (Harbin, China) using a light microscope. The staining score was defined by using intensity (0, negative; 1, weak; 2, moderate; 3, strong) and area (0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; 4, >75%). The final score was generated by assessing the percentage of area stained multiplied by the intensity of staining.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from tissues and cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using TransScript Reverse Transcriptase (AT101-02; Beijing Transgen Biotech Co., Ltd., Beijing, China). qPCR was performed using the SYBR Green

PCR Mix (AQ141-01; Beijing Transgen Biotech, Beijing, China) and the Bio-Rad CFX96™ Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers used were as follows: SIRT4, 5'-ATGAAGATGAGCTTTGCGT-3' (forward) and 5'-TCAGCATGGGTCATCAAAGG-3' (reverse); GAPDH, 5'-ATGGGGAAGGTGAAGGTCG-3' (forward) and 5'-GGGGTCATTGATGGCAACAATA-3' (reverse). GAPDH was used as an endogenous control. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec and 61°C for 30 sec. The analysis of RT-qPCR results according to the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (36).

**Western blot analysis.** Total protein was extracted from cells and tissues using a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Beyotime Institute of Biotechnology, Shanghai, China). Protein determination was performed using the BCA Protein assay kit (Takara Bio, Inc., Otsu, Japan) and proteins (25  $\mu$ g) were separated by SDS-PAGE (10% gels) and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) and blocked in 5% bovine serum albumin (Beyotime Institute of Biotechnology) was diluted in PBS for 2 h at room temperature. Membranes were incubated with anti-SIRT4 (catalog no. ab10140; 1:300, Abcam) or mouse anti- $\beta$ -actin (catalog no. sc-58673, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies at 4°C overnight, followed by incubation with Rabbit Anti-goat IgG H&L (catalog no. ab6697; 1:5,000; Abcam) or goat anti-mouse IgG H&L (catalog no. ab6708; 1:5,000; Abcam) for 1 h at room temperature. The protein bands were visualized by enhanced chemiluminescence (ECL; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) via FluorChem M imaging system (FCM, FM0422; ProteinSimple, San Jose, CA, USA) and analyzed using the Image J software (<https://imagej.nih.gov/ij/>).

**Apoptosis analysis.** Cell apoptosis was evaluated by using Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Briefly, a total of 1x10<sup>6</sup> HCT116 cells were collected and washed with PBS, and then the cells were resuspended with 1-ml Binding Buffer. 100  $\mu$ l solution (1x10<sup>5</sup>), 5  $\mu$ l PE Annexin V and 5  $\mu$ l 7-AAD were added in a 5 ml tube. The cells were vortexed gently and then incubated for 10 min in the dark at room temperature. At last, 400  $\mu$ l Binding Buffer was added in each tube before analyzed by flow cytometry. The flow cytometry data were analyzed by software (FlowJo 7.6.1; FlowJo LLC, Ashland, OR, USA).

**Cell viability assay.** Cellular viability was evaluated using CellTiter-Glo assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, HCT116 cells in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel) were plated in 96-well plates at a density of 1x10<sup>4</sup> cells/well at room temperature. At 0, 24, 48 and 72 h, 100  $\mu$ l CellTiter-Glo reagent was added into each well and mixed completely using an orbital shaker at room temperature. Following incubation for 10 min at room temperature, the luminescence signal was assessed using a luminometer.

*Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9)-mediated knockout of SIRT4 in HCT116 cells.* In the present study a CRISPR-Cas9 vector, targeting specific region of SIRT4 (5'-CCGAATCGGGGATACCAGAC-3') was used. Guide RNA sequence for CRISPR/Cas9 was designed using the CRISPR Design Tool (<http://tools.genome-engineering.org>) on August 2016. HCT116 cells were grown to 80-90% confluence and transfected with CRISPR plasmids (pSpCas9) by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. Cells were treated with 2  $\mu$ g/ml puromycin (ST551, Beyotime Institute of Biotechnology, Shanghai, China) for 24 h. Transfected cells were sorted to obtain single cell clones. Western blot analysis was used to confirm the knockout of SIRT4 in HCT116 cells.

*Invasion and migration assay.* Cell migration was evaluated using a Transwell chamber without (3422, Corning, USA) according to the manufacturer's protocol. Cell invasion was evaluated using a Transwell chamber with Matrigel (Corning Incorporated, Corning, NY, USA) according to the manufacturer's protocol. For the upper chambers,  $3 \times 10^5$  cells/ml were suspended in 0.5 ml of serum-free DMEM. The lower chamber contained DMEM supplemented with 10% FBS. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. At 48 h, cells remaining on the upper membrane surface were removed and the invasive cells on the lower surface were fixed with 1% crystal violet for 30 min and counted at room temperature. Images were captured using an Olympus inverted microscope (Olympus Corporation, Tokyo, Japan). The number of invaded cells was counted using ImageJ software (National Institutes of Health, MD, Bethesda, USA).

*5-FU treatment.* 5-fluorouracil 0.25 g/10 ml or oxaliplatin 50 mg was purchased from Harbin Medical University Cancer Hospital and storage at 4°C. HCT116 cells were treated with 5-FU for 36 h at 37°C and treated HCT116 cells with oxaliplatin for 48 h at 37°C.

*Statistical analysis.* Data were analyzed using GraphPad Prism (version 5.0; GraphPad Software, Inc.). Data are expressed as the mean  $\pm$  standard deviation. Three individual experiments were performed. Results were analyzed using Student's t-test or one-way ANOVA analysis, following the analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Expression of SIRT4 in CRC based on data obtained from TCGA.* The SIRT4 expression data from 174 samples (including 19 normal colon tissues and 155 CRC tissues) were downloaded from TCGA data portal. The results demonstrated that the expression of SIRT4 was significantly decreased in colon adenocarcinoma tissues compared with normal colon tissues (Fig. 1).

*SIRT4 is downregulated in CRC cell lines and tissues.* mRNA and protein expression levels of SIRT4 were examined in CRC cell lines and tissues. The results confirmed that mRNA expression

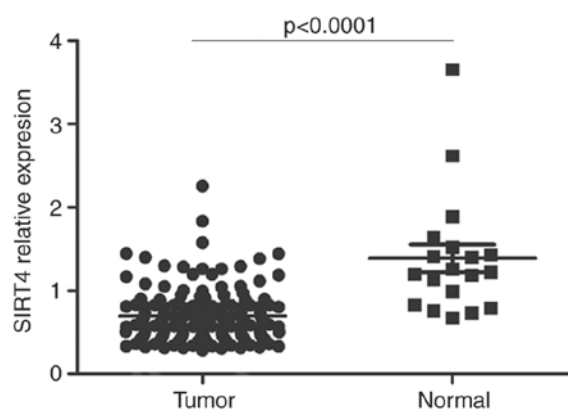


Figure 1. The expression of SIRT4 was evaluated in CRC and normal tissues using data obtained from TCGA. SIRT, sirtuin; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas.

levels of SIRT4 were significantly decreased compared with that in adjacent normal tissues (Fig. 2A). Additionally, mRNA expression levels of SIRT4 were decreased in CRC cell lines (HCT116, SW1116, SW620 and DLD1) compared with that in the normal colorectal cell line FHC (Fig. 2B). Protein expression of SIRT4 was evaluated in tissues using western blot analysis and immunohistochemistry. The results demonstrated that the expression level of SIRT4 in CRC tissues was decreased compared with that in adjacent normal tissues (Fig. 2C-E). Thus, SIRT4 is downregulated in CRC tissues and cells, suggesting that SIRT4 may be involved in the pathogenesis of CRC.

*SIRT4 functions as a potential tumor suppressor by repressing the proliferation, and migration of CRC cells.* To determine the function of SIRT4 in colorectal cancer, CRISPR/Cas9-mediated knockout of SIRT4 was performed in HCT116 cells. The knockout of SIRT4 was confirmed using western blot analysis (Fig. 3A). The proliferative ability of control cells (HCT116 SIRT4<sup>+/+</sup>) and SIRT4 knockout cells (HCT116 SIRT4<sup>-/-</sup>) was evaluated. The results demonstrated that HCT116 SIRT4<sup>-/-</sup> cells grew faster compared with HCT116 SIRT4<sup>+/+</sup> cells (Fig. 3B). Additionally, the effect of SIRT4 knockout on the migratory abilities was determined in HCT116 cells. The results demonstrated that HCT116 SIRT4<sup>-/-</sup> significantly enhanced the migratory ability (Fig. 3C) and invasive ability (Fig. 3D). Therefore, the knockout of SIRT4 promoted the proliferation, migration and invasion of HCT116 cells.

*Knockout of SIRT4 decreases chemosensitivity in CRC cells by inhibiting apoptosis.* The effects of SIRT4 knockout in regulating chemotherapy in CRC were also evaluated. HCT116 SIRT4<sup>-/-</sup> and HCT116 SIRT4<sup>+/+</sup> were treated with various concentrations of 5-fluorouracil (5-FU) or oxaliplatin for 48 h at 37°C. CellTiter-Glo Luminescent assay was employed to determine the effect of SIRT4 on the chemosensitivity of CRC cells. The results demonstrated that the viability of HCT116 SIRT4<sup>-/-</sup> cells was increased compared with that in SIRT4<sup>+/+</sup> in response to treatment with 5-FU or oxaliplatin by inhibition percent (%) (Fig. 4A and B). These results suggest that knockout of SIRT4 may decrease the chemosensitivity of CRC cells. Next, the effect of SIRT4 knockout on cells apoptosis was evaluated. Due to the high drug concentration required

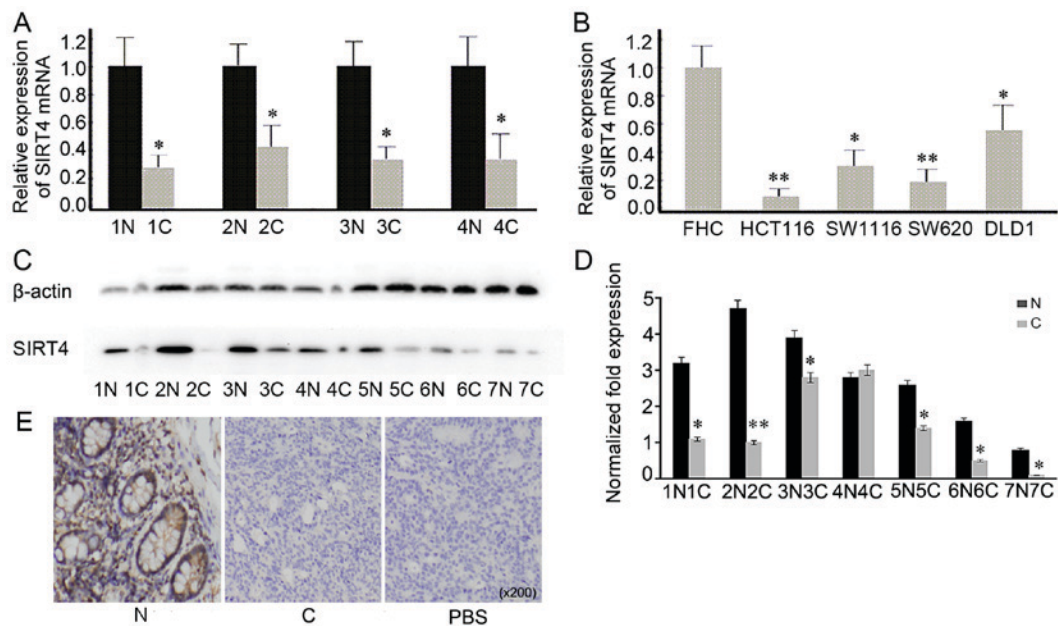


Figure 2. Detection of SIRT4 expression in CRC tissues and cell lines. (A) mRNA expression levels of SIRT4 in representative CRC tissues and adjacent normal tissues was examined. \* $P < 0.05$  vs. N. (B) mRNA expression levels of SIRT4 in CRC cell lines (HCT116, SW1116, SW620 and DLD1) was decreased compared with that in the normal cell line FHC. \*\* $P < 0.01$ , \* $P < 0.05$  vs. FHC. (C) The expression of SIRT4 in seven CRC tissues and adjacent normal tissues was evaluated using western blot analysis. (D) The densitometric analysis of SIRT4 expression protein was downregulated in CRC tissues compared with that in normal tissues. \*\* $P < 0.01$ , \* $P < 0.05$  vs. N. (E) Representative immunohistochemical staining of SIRT4 in human CRC and adjacent normal tissues. SIRT4 was mainly expressed in the cytoplasm, and its expression was decreased in CRC tissues compared with that in adjacent normal tissue. SIRT, sirtuin; CRC, colorectal cancer; N, normal tissue; C, CRC tissue; PBS, negative control.

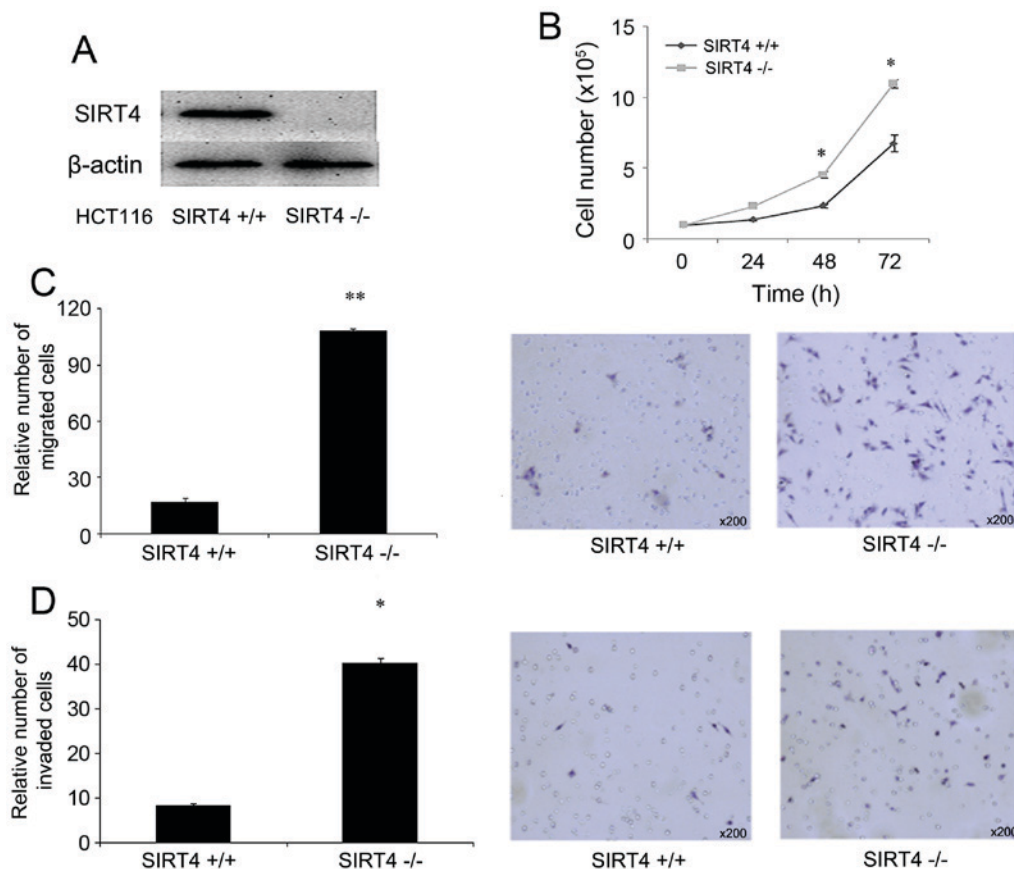


Figure 3. SIRT4 regulates proliferation and migration in CRC cells. (A) Knockout of SIRT4 in HCT116 cells was confirmed by western blot analysis. (B) HCT116 SIRT4<sup>-/-</sup> cells exhibited a significant increase in proliferation. \* $P < 0.05$  vs. HCT116 SIRT4<sup>-/-</sup>. (C) Knockout of SIRT4 promoted migration of HCT116 cells by counting numbers of migrating cells (magnification, x200). \*\* $P < 0.01$  vs. HCT116 SIRT4<sup>-/-</sup>. (D) Knockout of SIRT4 promoted invasion of HCT116 cell by counting numbers of invasive cells (magnification, x200). \* $P < 0.05$  vs. HCT116 SIRT4<sup>-/-</sup>. SIRT, sirtuin; CRC, colorectal cancer; HCT116 SIRT4<sup>+/+</sup>, wild-type; HCT116 SIRT4<sup>-/-</sup>, SIRT4 knockout.

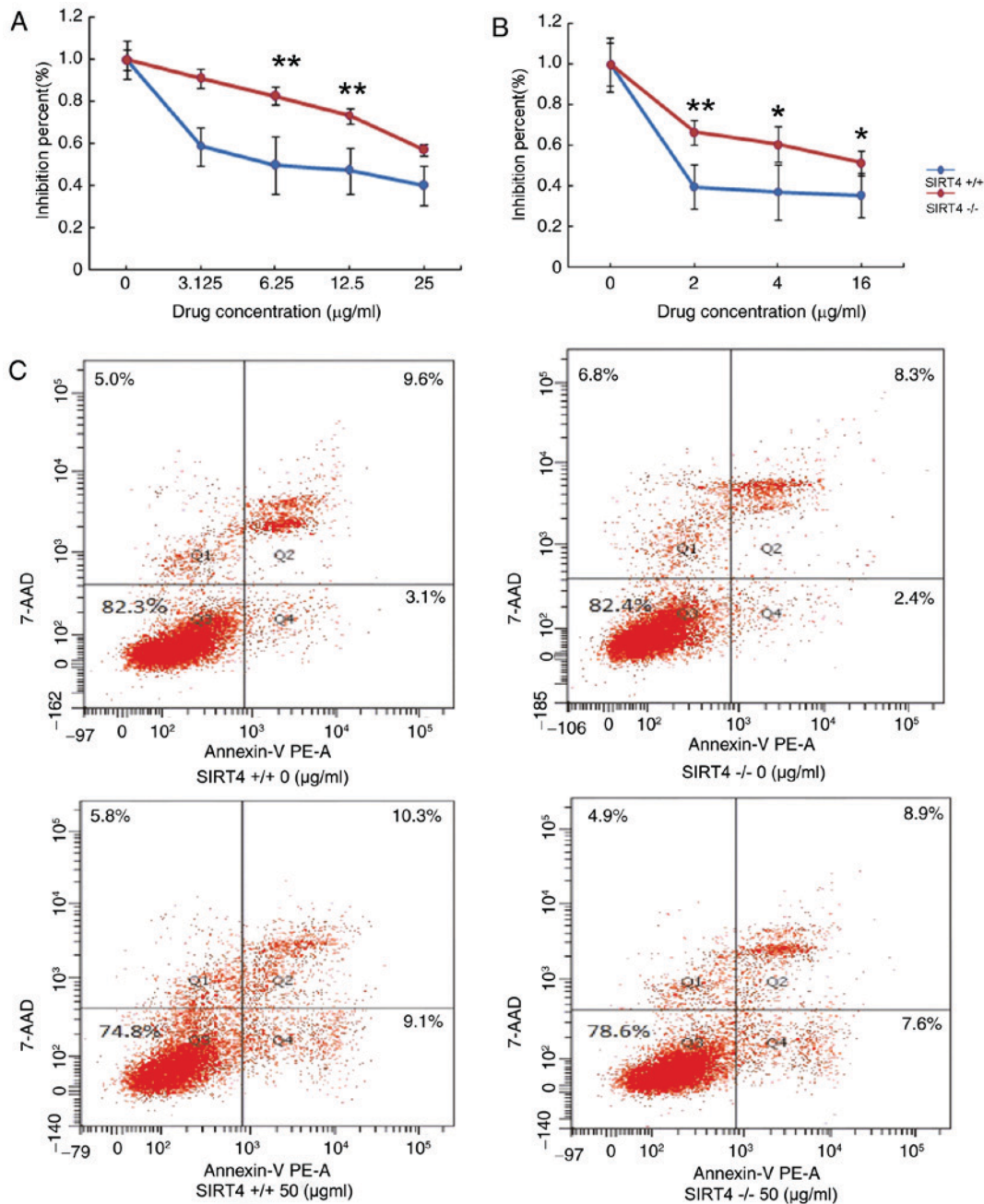


Figure 4. Knockout of SIRT4 decreases the chemotherapeutic sensitivity of CRC cells to 5-FU or oxaliplatin by inhibiting apoptosis. (A) The viability of HCT116 SIRT4<sup>-/-</sup> cells is decreased compared with HCT116 SIRT4<sup>+/+</sup> in response to 5-FU treatment by evaluating inhibition percent (%) \*\*P<0.01 vs. HCT116 SIRT4<sup>-/-</sup>. (B) The viability of HCT116 SIRT4<sup>-/-</sup> cells is decreased compared with HCT116 SIRT4<sup>+/+</sup> in response to oxaliplatin treatment by evaluating inhibition percent (%), \*\*P<0.01 vs. HCT116 SIRT4<sup>-/-</sup>, \*P<0.05 vs. HCT116 SIRT4<sup>-/-</sup>. (C) SIRT4 knockout inhibits apoptosis in response to 5-FU. SIRT, sirtuin; HCT116 SIRT4<sup>+/+</sup>, wild-type; HCT116 SIRT4<sup>-/-</sup>, SIRT4 knockout; 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin; 5-FU, 5-fluorouracil.

for apoptosis, HCT116 SIRT4<sup>-/-</sup> cells and HCT116 SIRT4<sup>+/+</sup> cells were treated with 50 µg/ml 5-FU and cell apoptosis was evaluated using flow cytometry. The results demonstrated that the apoptosis rate was decreased in HCT116 SIRT4<sup>-/-</sup> cells compared with that of HCT116 SIRT4<sup>+/+</sup> cells (Fig. 4C), suggesting that knockout of SIRT4 may decrease sensitivity to chemotherapy by inhibiting apoptosis in CRC cells.

## Discussion

Previous studies demonstrated that SIRT4 is downregulated in various cancer types (23,24) and is associated with

multiple cancer biological behaviors (23). However, the function of SIRT4 in CRC remains unclear. The results of the present study revealed that the expression of SIRT4 was significantly decreased in CRC tissues and cell lines at mRNA and protein levels, which is consistent with previous studies. Previous studies revealed that SIRT4 might function as a tumor suppressor. Jeong *et al* (24) demonstrated that SIRT4 may suppress tumor formation by inhibiting glutamine metabolism, and overexpression of SIRT4 may prevent the growth of HeLa cells. Additionally, Csibi *et al* (23) revealed that overexpression of SIRT4 may prevent the growth of the prostate cancer cell line DU145 and the colon

cancer cell line DLD-1. Jeong *et al* (32) revealed that SIRT4 might inhibit the growth of Myc-induced B lymphoma cell by inhibiting glutamine metabolism. In the present study, stable human SIRT4 KO cells were established and the effects of knockout of SIRT4 on proliferation, migration and invasion of CRC cells were examined. The results demonstrated that knockout of SIRT4 increased the proliferation, migration and invasion of CRC cells, thus confirming the findings of previous studies (30).

Targeting SIRT4 may be a novel therapeutic approach in CRC. Chemotherapy is considered as one of the most effective treatment approaches in CRC (37). 5-FU and oxaliplatin are widely used for the treatment of CRC (38). 5-FU-based chemotherapy in combination with oxaliplatin or irinotecan is used for the treatment of metastatic CRC (39). 5-FU metabolites are incorporated into DNA to suppress cell growth (40). In tumor cells, 5-FU is metabolized to cytotoxic compounds, which bind to thymidylate synthase in order to repress DNA synthesis (41). Oxaliplatin, a platinum-based chemotherapeutic drug, containing 1,2-diaminocyclohexane carrier ligand, forms platinum-DNA adducts, which block DNA replication. In addition, oxaliplatin has demonstrated high efficacy against tumor growth both *in vitro* and *in vivo* (42). Oxaliplatin treatment combined with 5-FU increases progression-free survival and overall survival of CRC (43). Oxaliplatin and 5-FU may act synergistically to regulate thymidylate synthase (44). In the present study, the effects SIRT4 on the chemosensitivity of CRC cells were evaluated. To the best of our knowledge, this is the first study to examine the role of SIRT4 in cancer chemotherapy. The results demonstrated that SIRT4 KO cells exhibited increased viability compared with control cells, suggesting that SIRT4 may enhance the chemosensitivity of CRC cells to 5-FU and oxaliplatin. Next, the underlying molecular mechanism was investigated. A number of events involved in apoptosis are closely associated with mitochondria, including the release of caspase activators, loss of mitochondrial transmembrane potential, altering electron transport, and participation of Bcl-2 family proteins (45,46). SIRT4 is located in the mitochondria. Previous studies have demonstrated that SIRT4 serves an important function in hypoxia-induced apoptosis in H9c2 cardiomyoblast cells through affecting Bcl-2-associated-X protein (Bax) translocation (47). Therefore, it was hypothesized that SIRT4 may affect the chemotherapeutic sensitivity of CRC cells through regulating apoptosis. The results revealed that SIRT4 knockout prevents the apoptosis of CRC cells in response to 5-FU. These results suggest that SIRT4 may act as a tumor suppressor and may be used as a novel therapeutic target in CRC.

In conclusion, the present study demonstrated that the expression of SIRT4 is significantly decreased in CRC tissues at mRNA and protein levels, and that SIRT4 knockout increases tumor proliferation, migration and invasion, thus suggesting that SIRT4 may act as a tumor suppressor. Additionally, SIRT4 knockout affects the chemotherapeutic sensitivity of CRC cells by regulating apoptosis. The present study provides a promising target for CRC therapy.

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#### Availability of data and materials

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YaZ conceived and designed the experiments. YuZ, GW, XL, TW and MW performed the experiments and analyzed the data. YuZ and YaZ wrote the paper. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All patients provided written informed consent prior to their inclusion and the present study was approved by the Ethics Committee of Harbin Medical University (Heilongjiang, China).

#### Consent for publication

This research was completed in compliance with the Helsinki Declaration. The patients provided written informed consent for the publication of the data. The data collection and analysis were carried out without disclosing the identities of the patients.

#### Competing interests

The authors declare that they have no competing interests.

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